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Measurement of dipolar couplings in a transducin peptide fragment weakly bound to oriented photo-activated rhodopsin

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Abstract

Rhodopsin-containing disks, isolated from rod outer segments of bovine retina, align at high magnetic fields with their membrane normal parallel to the magnetic field. After light-activation of rhodopsin, transient binding of the C-terminal transducin undecapeptide, selectively labeled with ^{15}N at Leu^5 and Gly^9, results in residual dipolar contributions to the $^1J_{NH}$ splittings for these two residues. Both residues show $^1J_{NH}$ splittings which are smaller than in the dark-adapted or rhodopsin-free sample, and return to their isotropic values at a rate determined by the decay of the meta II state of rhodopsin. The dipolar couplings indicate that in the bound state, N-H vectors of Leu^5 and Gly^9 make angles of 48±4° and 40±8°, respectively, with the disk normal. These 'transferred' dipolar couplings potentially offer a useful method for studying the conformation and orientation of flexible, low affinity ligands when bound to oriented integral membrane receptors.

Introduction

G protein-coupled receptors play a key role in regulating a diverse array of cellular processes. Knowledge of their structure and their mode of interaction with ligand proteins is therefore of pivotal importance for understanding intercellular signaling and for development of drugs which can modulate this activity. Although important information on the structure of G proteins and their receptors has become available in recent years (Hamm, 1998), mainly as a result of advances in crystallography (Wall et al., 1995; Lambright et al., 1996), electron diffraction (Schertler et al., 1993, 1995), EPR (Farrens et al., 1996), and NMR (Shieh et al., 1997; Yeagle et al., 1997a,b), a detailed atomic view of a G protein–receptor complex has remained out of reach. Information on the conformation of G protein peptide fragments when bound to such integral membrane proteins has been obtained from solution state transferred NOE (TrNOE) experiments (Dratz et al., 1993; Kisselev et al., 1998), but the rapid spin diffusion occurring when such a ligand is tightly bound to the extremely large protein–membrane complex could interfere with quantitative analysis of this type of data. Also, this type of TrNOE experiment does not provide any information on the orientation of the ligand relative to the membrane protein. Here, we demonstrate that in favorable cases it is possible to measure dipolar couplings for a ligand which is in rapid exchange between free solution and a state where it is ligated to a receptor protein, which is anchored in a magnetically oriented membrane bilayer. Such measurements offer information both on the conformation of the bound ligand and on its orientation relative to the oriented bilayer.

Experimental procedure

Experiments are demonstrated for an 11-residue synthetic transducin peptide fragment (IRENLKDSGLF), here referred to as S2, which is ^{15}N-labeled in two positions, Leu^5 and Gly^9. The amino acid sequence of S2 closely resembles the C-terminus of the transducin α-subunit (residues 340–350), which is one of the three regions known to interact with rhodopsin (Hamm et al., 1988). The 40 kDa integral membrane protein rhodopsin constitutes a well characterized prototype of the superfamily of G protein-coupled receptors. The meta II state of light-activated rhodopsin binds the heterotrimeric G protein transducin (G_t) (Kibelbek et al., 1991) and catalyzes the GDP-GTP exchange reaction on the bound ligand. In this process, the $G_t\alpha{\cdot}GTP$ subunit dissociates from the $G_t\beta\gamma$ fragment, and activates downstream effectors. The C-terminus of the α-subunit of G proteins is an essential determinant of the specificity of receptor/G protein coupling (Conklin et al., 1993). Although the last 7 residues of $G_t\alpha$ are disordered in the crystal structure of the heterotrimer $G_t\alpha\beta\gamma$ (Lambright et al., 1996), solution state TrNOE experiments on peptide analogs indicate they adopt a distinct conformation upon receptor binding (Dratz et al., 1993; Kisselev et al., 1998). The S2 peptide differs in two amino acids from the sequence of $G_t\alpha$(340–350). Replacement of Lys^{341} with Arg has no significant influence on the characteristics of peptide binding to rhodopsin but was used for compatibility with the TrNOE data of Dratz et al. (1993), who used the same substitution. Cys^{347} was replaced by Ser, to increase the peptide off-rate. Both the peptide off-rate and the observed magnitude of the residual dipolar couplings increase with temperature over the studied range from 10 to 25 °C. However, the lifetime of the peptide-binding meta II state of rhodopsin decreases with rising temperature. Therefore, as a compromise, dipolar couplings were measured at 20 °C.

All experiments were conducted using 270 μl Shigemi microcells at either 600 or 800 MHz ^{1}H frequency on Bruker Avance model NMR spectrometers. Samples contain 5 mg/ml rhodopsin rich disk-membranes (50% w/w rhodopsin), i.e. 63 μM rhodopsin, and a 41-fold excess of the S2 peptide (2.6 mM), in 90% H_2O, 10% D_2O, pH 6.6. Two identical samples were prepared from the same stock solution for measurements at 600 MHz and 800 MHz ^{1}H frequency, respectively.

Intact disk-membranes were isolated from rod outer segments (ROS) of bovine retina as described elsewhere (Smith and Litman, 1982). Samples were kept on ice at 0 °C and all manipulations were performed in the dark or under dim red light, in order to avoid premature bleaching of rhodopsin (and subsequent decay to opsin). Prior to transfer into the sample tube, disks were gently monomerized by multiple (30 times) pipetting of the solution through a 150 μm pipet tip. This greatly reduces the number of large lumps of loosely aggregated disks (grape-like structures) as observed by light microscopy. As a result, alignment of the disks increased, as monitored by the quadrupole splitting of the ^{2}H lock signal (ca. 5 Hz at 20 mg disks per ml) and ^{31}P NMR spectra of the lipids. ^{31}P spectra were recorded on the 600 MHz spectrometer using 20 mg disks per ml. Most of the inhomogeneously broadened ^{31}P resonance intensity occurred at 23 ppm downfield of inorganic phosphate (data not shown). In a static phospholipid ^{31}P powder pattern, the most downfield shoulder at about 27 ppm corresponds to phospholipid oriented with its long axis parallel to the magnetic field, and the upfield singularity, corresponding to phospholipid orthogonal to the field, occurs at about −15 ppm. The ^{31}P resonance position of 23 ppm observed for ROS-disks indicates nearly full alignment at 14 Tesla with the membrane normal parallel to the field. This is similar to what was previously observed for bacteriorhodopsin containing purple membrane fragments (Lewis et al., 1985; Koenig et al., 1999). The positive anisotropy of the magnetic susceptibility of ROS-disks is dominated by that of the rhodopsin transmembrane helices (rhodopsin accounts for about 50% w/w of the disks), and perhaps by the large fraction of double bonds in the lipid hydrocarbon chains (50% of the chains are docosahexaenoic acids with 6 *cis*-double bonds; Stinson et al., 1991).

Results and discussion

Initial measurements on the native $G_t\alpha$(340–350) peptide at 2 °C, without the C347S substitution, gave high quality transferred NOE spectra, albeit with some evidence of spin diffusion (data not shown). Measurement of ^{15}N T_2 values on the free peptide before and after exposing rhodopsin samples containing a 65-fold excess of peptide to an intense light source for 40 s shows an increase in the ^{15}N transverse relaxation rate from 5±0.5 s^{-1} to 8.9±0.5 s^{-1} for four ^{15}N labeled

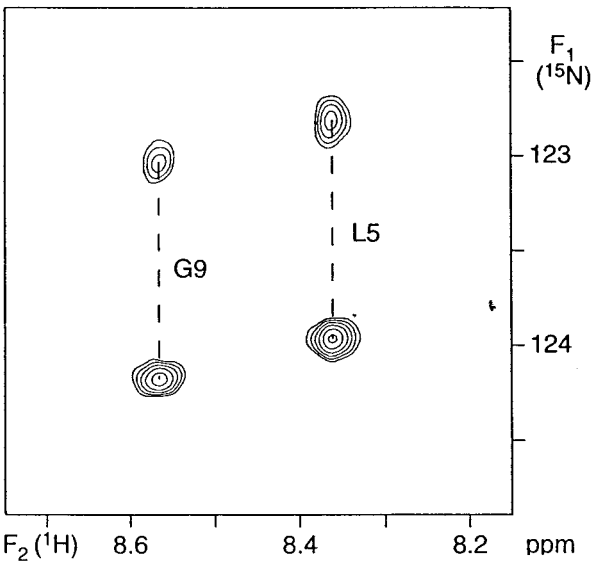

Figure 1. 800 MHz two-dimensional HSQC spectrum without ^{1}H decoupling in the t_1 dimension for the ^{15}N-L/G S2 peptide (2.6 mM) in the presence of 5 mg/ml rhodopsin-containing disks (63 μM rhodopsin). The 9-min HSQC spectrum was started 2 min after exposing the sample to bright light. Spectra were processed and analyzed with the program NMRPipe (Delaglio et al., 1995). The acquisition times were 120 ms (t_1) and 57 ms (t_2). Data were apodized with 72°-shifted sine bell (t_1) and 72°-shifted squared sine bell (t_2) windows, prior to zero filling and Fourier transformation.

amides. However, no change in the $^{1}J_{NH}$ couplings was observed, suggesting that the peptide is in the slow exchange limit, i.e., during a single binding event any ^{15}N transverse magnetization completely decays before the peptide dissociates from rhodopsin. This slow exchange regime is confirmed by the absence of any increase in the N-H dipolar/^{15}N CSA cross correlation relaxation rate (Goldman, 1984; Tjandra et al., 1996) when binding of peptide to rhodopsin is triggered by photo-activation.

Subsequent measurements were carried out on the S2 peptide. At 3 °C, an increase in $^{1}H^{N}$ line width upon photoactivation was observed for several resonances, which suggests an increase in the off-rate relative to the native $G_t\alpha(340–350)$ peptide. However, the absence of significant changes in dipolar coupling, or in the observed N-H dipolar/^{15}N CSA cross correlation rate, indicates that the off-rate remains too low for measuring dipolar couplings in the rhodopsin-bound form. In contrast, after raising the temperature to 20 °C, considerable $^{1}H^{N}$ line broadening (ca. 15 Hz) is observed, with a concomitant increase in the N-H dipolar/^{15}N CSA cross correlation rate (Figure 2). A series of 10 9-min F_1-coupled ^{1}H-^{15}N HSQC spec-

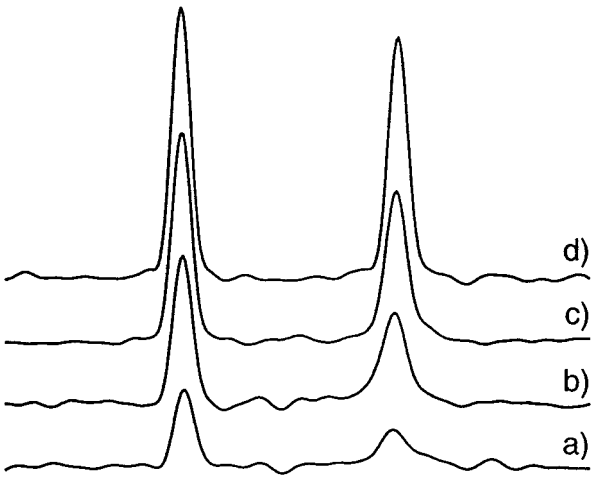

Figure 2. F_1 cross sections through the Gly^{9} resonance for four 800 MHz ^{1}H-coupled HSQC spectra of the S2 peptide in the presence of 5 mg/ml ROS disks. The four HSQC experiments (9 min each) were started (a) 2, (b) 11, (c) 20, and (d) 84 min after exposing the sample to bright light. The fraction of meta II state rhodopsin, which binds the S2 peptide, decreases exponentially with time, resulting in an increase of the Gly^{9} ^{15}N-{^{1}H} doublet intensity, and a decrease in cross-correlation induced doublet asymmetry. Based on the signal-to-noise ratio and the line width, the error in the derived splittings (using parabolically interpolated peak picking) can be estimated from a graph presented by Kontaxis et al. (in press). This yields error estimates of 0.5 Hz for (a) and 0.1 Hz for (d), in agreement with the experimental reproducibility of these measurements.

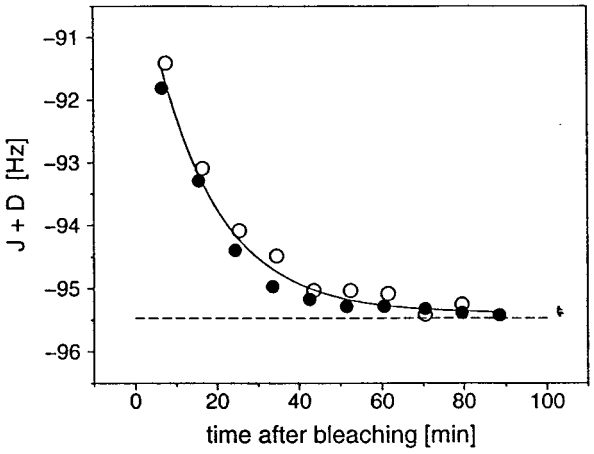

Figure 3. Measured Gly^{9} J_{NH} splitting in the HSQC spectra recorded at 600 MHz (open circles) and 800 MHz (filled circles) as a function of time after bleaching of rhodopsin. The horizontal dashed line corresponds to the J_{NH} splitting measured in the free S2 peptide, in the absence of ROS disks. The time axis corresponds to the mid-point of the HSQC experiments, i.e., 4.5 min after the start of the experiments.

tra were recorded, starting 2 min after exposing the sample to light. The first spectrum, which has considerably weaker intensity than the later ones as a result of the increased ^{1}H line width shortly after light exposure, is shown in Figure 1. F_1 cross sections through four of the HSQC spectra for the amide of Gly^9 are shown in Figure 2. Clearly, at early times pronounced N-H dipolar/^{15}N CSA cross correlation is observed, and signals are considerably weaker than in the later spectra, where the peptide-binding meta II state has decayed by converting to meta III and finally to opsin.

Measurement of the $^1J_{NH}$ splitting (Figure 3) shows a clear dependence on the time after illumination. Shortly after light exposure, the $^1J_{NH}$ splitting for both Leu^5 and Gly^9 is smaller than the value measured in dark-adapted rhodopsin, which is very similar to that in the free peptide (to within 0.3 Hz), and the value at a long delay after illumination. This change in J splitting results from the dipolar coupling contribution, $^1D_{NH}$, which does not average to zero when the peptide is bound to the magnetically aligned rhodopsin-containing disks. Conceptually, this is similar to the pioneering work of Prestegard and co-workers (Tolman et al., 1995), who observed that in paramagnetic myoglobin weak alignment with the magnetic field results in a dipolar contribution to the $^1J_{NH}$ splitting. Subsequently it has been shown that orientation of a protein can also be induced by dissolving it in a dilute, magnetically ordered liquid crystalline phase (Bax and Tjandra, 1997; Tjandra and Bax, 1997; Prestegard, 1998). In contrast to the present study, most of these liquid crystal studies focus on well structured proteins. Exceptions have been two cases studied by the Prestegard group. In the first study, one half of a flexibly linked two-domain protein was found to transiently bind to phospholipid bicelles (Fischer et al., 1999). In a second study, more closely related to our present work, the conformation of a ligand was studied when transiently bound to a macromolecule, which itself is weakly oriented by the liquid crystal (Bolon et al., 1999). In our present study, orientation of the flexible ligand is induced by transient binding to the highly aligned receptor.

After photo-bleaching, the peptide-binding meta II state of rhodopsin decays irreversibly in vitro. As a result, $^1D_{NH}$ decays exponentially to zero as a function of time after bleaching (Figure 3), with a fitted time constant of 16±2 min. Exponential decay times of 14±2 min (with S2 peptide) and 11±2 min (without peptide) were obtained spectrophotometrically for the meta II to meta III transition under the same conditions (20 °C; pH 6.7). This observed decay time is somewhat longer than the 8 min reported by Kibelbek et al. (1991) for this transition at pH 8. Slower decay at lower pH agrees with the base catalyzed nature of the decay of meta II (Blazynski and Ostroy, 1984).

Both in the dark-adapted case and in the fully decayed sample (long after illumination), the $^1J_{NH}$ splittings are virtually the same as in the free peptide sample. This indicates that steric alignment of the peptide by the disks or by transient non-specific binding to the oriented disks is negligible.

Assuming that there is only a single binding mode for the peptide to rhodopsin (i.e., all bound peptides are oriented in the same manner with respect to the bilayer normal), the molecular alignment tensor for the peptide is axially symmetric. Then, the observed dipolar coupling is given by:

$$^1D_{NH} = -\mathbf{S}(\mu_0/4\pi)([Pb]/[Pf])\gamma_N\gamma_H h \times (3\cos^2\theta - 1)/(4\pi^2 r_{NH}^3) \quad (1)$$

where [Pb]/[Pf] is the ratio of bound versus free peptide, θ is the angle between the N-H vector and the magnetic field, and **S** is the order parameter, including both the effect of incomplete alignment of the disk when integrated over its entire surface and internal mobility in the bound form. All other constants have their usual meaning. In order to obtain an accurate value for θ from the measured $^1D_{NH}$ value, **S** ([Pb]/[Pf]) must be known. In protein liquid crystal studies, this constant can be estimated from the distribution of the observed dipolar couplings (Clore et al., 1998). However, in the present case, with only two dipolar couplings available, this is not possible. Nevertheless, in order to illustrate the use of such couplings, a rough estimate of [Pb]/[Pf] can be obtained from the observed ca 15 Hz ^{1}H line broadening of the amide protons at the time the first HSQC spectrum is recorded, a few minutes after bleaching. If we assume the $^1H^N$ line width in the bound state to be 20 kHz, this yields [Pb]/[Pf] = 0.00075. For an assumed **S** = 2/3, this then yields $(3\cos^2\theta - 1) = {}^1D_{NH}$ / 5.7 Hz. However, it must be emphasized that there is considerable (at least twofold) uncertainty in the denominator, resulting from the uncertainty in the **S** ([Pb]/[Pf]) value, used to derive this number.

The first HSQC spectrum after bleaching yields $^1D_{NH}$ values of −1.5 Hz (Leu^5) and -3.2 Hz (Gly^9). These correspond to θ values of 48±4° (Leu^5) and 40±8° (Gly^9), where the errors are dominated by the estimated 50% uncertainty in **S** ([Pb]/[Pf]). Despite the large uncertainty in **S** ([Pb]/[Pf]), the errors in

the derived angles are relatively modest. With full labeling of the peptide, considerably better estimates for **S** ([Pb]/[Pf]) should be possible on the basis of the observed range of couplings, further reducing the uncertainties in derived bond angle orientations.

Concluding remarks

We have shown that it is possible to derive angular information from dipolar couplings measured for a ligand which is transiently bound to an intact integral membrane receptor protein. These angular constraints should be useful complements to distance information derived from transferred NOE experiments (Clore and Gronenborn, 1982; Ni, 1994) and, in addition, provide the orientation of the ligand relative to the membrane normal. A limitation of the method is that it is only applicable to ligands with high off-rates ($>5 \times 10^4$ s^{-1}), i.e., to ligands which bind in the millimolar range. Use of deuterated peptides can improve this situation by as much as fivefold, but decreases the number of dipolar interactions accessible in such experiments. The requirement of a high off-rate applies both to the transferred dipolar coupling and to the transferred NOE experiment. Although the latter experiment yields spectra with narrow resonances when applied to much tighter peptide–rhodopsin complexes, bound times longer than ca 100 μs result in extensive spin diffusion during a single binding event.

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References

Bax, A. and Tjandra, N. (1997) *J. Biomol. NMR*, **10**, 289–292.
Blazynski, C. and Ostroy, S.E. (1984) *Vision Res.*, **24**, 459–470.
Bolon, P.J., Al-Hashimi, H.M. and Prestegard, J.H. (1999) *J. Mol. Biol.*, **293**, 107–115.
Clore, G.M. and Gronenborn, A.M. (1982) *J. Magn. Reson.*, **48**, 402–417.
Clore, G.M., Gronenborn, A.M. and Bax, A. (1998) *J. Magn. Reson.*, **133**, 216–221.
Conklin, B.R., Farfel, Z., Lustig, K.D., Julius, D. and Bourne, H.R. (1993) *Nature*, **363**, 274–276.
Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J. and Bax, A. (1995) *J. Biomol. NMR*, **6**, 277–293.
Dratz, E.A., Furstenau, J.E., Lambert, C.G., Thireault, D.L., Rarick, H., Schepers, T., Pakhlevaniants, S. and Hamm, H.E. (1993) *Nature*, **363**, 276–281.
Farrens, D.L., Altenbach, C., Yang, K., Hubbell, W.L. and Khorana, H.G. (1996) *Science*, **274**, 768–770.
Fischer, M.W.F., Losonczi, J.A., Weaver, J.L. and Prestegard, J.H. (1999) *Biochemistry*, **38**, 9013–9022.
Goldman, M. (1984) *J. Magn. Reson.*, **60**, 437–452.
Hamm, H.E. (1998) *J. Biol. Chem.*, **273**, 669–672.
Hamm, H.E., Deretic, D., Arendt, A., Hargrave, P.A., König, B. and Hofmann, K.P. (1988) *Science*, **241**, 832–835.
Kibelbek, J., Mitchell, D.C., Beach, J.M. and Litman, B.J. (1991) *Biochemistry*, **30**, 6761–6769.
Kisselev, O.G., Kao, J., Ponder, J.W., Fann, Y.C., Gautam, N. and Marshall, G.R. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 4270–4275.
Koenig, B.W., Hu, J.-S., Ottiger, M., Bose, S., Hendler, R.W. and Bax, A. (1999) *J. Am. Chem. Soc.*, **121**, 1385–1386.
Kontaxis, G., Clore, G.M. and Bax, A., *J. Magn. Reson.*, in press.
Lambright, D.G., Sondek, J., Bohm, A., Skiba, N.P., Hamm, H.E. and Sigler, P.B. (1996) *Nature*, **379**, 311–319.
Lewis, B.A., Rosenblatt, C., Griffin, R.G., Courtemanche, J. and Herzfeld, J. (1985) *Biophys. J.*, **47**, 143–150.
Ni, F. (1994) *Prog. NMR Spectrosc.*, **26**, 517–606.
Prestegard, J.H. (1998) *Nat. Struct. Biol.*, **5**, NMR Suppl., 517–522.
Schertler, G.F.X., Villa, C. and Henderson, R. (1993) *Nature*, **362**, 770–772.
Schertler, G.F.X. and Hargrave, P.A. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 11578–11582.
Shieh, T., Han, M., Sakmar, T.P. and Smith, S.O. (1997) *J. Mol. Biol.*, **269**, 373–384.
Smith, H.G. and Litman, B.J. (1982) *Methods Enzymol.*, **81**, 57–61.
Stinson, A.M., Wiegand, R.D. and Anderson, R.E. (1991) *Exp. Eye Res.*, **52**, 213–218.
Tjandra, N. and Bax, A. (1997) *Science*, **278**, 1111–1114.
Tjandra, N., Szabo, A. and Bax, A. (1997) *J. Am. Chem. Soc.*, **118**, 6986–6991.
Tolman, J.R., Flanagan, J.M., Kennedy, M.A. and Prestegard, J.H. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 9279–9283.
Wall, M., Coleman, D.E., Lee, E., Iniguez-Lluhi, J.A., Posner, J.A., Gilman, A.G. and Sprang, S.R. (1995) *Cell*, **83**, 1047–1058.
Yeagle, P.L., Alderfelder, J.L., Salloum, A.C., Ali, L. and Albert, A.D. (1997) *Biochemistry*, **36**, 3864–3869.
Yeagle, P.L., Alderfelder, J.L. and Albert, A.D. (1997) *Biochemistry*, **36**, 9649–9654.